

THE ISOLATION AND CHARACTERIZATION
OF A DNA FROM THE CHLOROPLASTS OF
PISUM SATIVUM

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

August, 1966

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. S. H. West, his supervisory chairman, for financial assistance and the use of the laboratory facilities. In addition, he would like to thank Mr. J. Brollman and D. Block for certain technical assistance, and Mr. E. B. Wilson for his aid in preparing the manuscript.

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INTRODUCTION

The presence of hereditary material within the cytoplasmic organelles has been a subject of debate for a long time. The presence of extranuclear Deoxyribose Nucleic Acid (DNA) has been extremely difficult to demonstrate due to the possibility of contamination from the nucleus or from external microbiological sources. Plastids are known to reproduce themselves. Numerous photomicrographs demonstrate this (Green, 1964). Chemical analysis showed the presence of DNA in chloroplast fractions but were open to the same objections as were raised against the reported occurrence of the molecule in the cytoplasm. Recent experiments with algae, particularly with aseptic cultures of the large unicellular alga, Acetabularia, an organism from which the nucleus may be excised, demonstrated that DNA was present in the plastid (Gibor and Izawa, 1963).

The inheritance of plastids and the control of their metabolism are subjects of general interest. The presence of DNA in the chloroplasts of algae raised the question as to whether it was present in the plastids of higher plants. Such a discovery would provide a molecular basis for the reported partial genetic independence of plastids. Any

further developments in the field of chloroplast genetics would require a knowledge of the amount of nucleic acids produced and the different types made in the plastid.

This work was begun with the hope of discovering DNA in the chloroplasts of higher plants. Since that time several investigators, Chun, Vaughan and Rich (1963), Kirk (1963) have reported the occurrence of DNA in plastids of spinach and broad bean. It was attempted to extend the work to the plastids of pea.

REVIEW OF THE LITERATURE

It is well known that the nucleus is the site where the major portion of the hereditary material is concentrated. Early in the history of the science of genetics it was suspected that plastids contained a genetic component independent of the nuclear one. Investigations on the inheritance of plastids in variegated and albino plants indicated that this was so even though the nucleus played a very large role in determining the phenotype of the plastid (Granick, 1955). Work using the flagellate, Euglena gracilis, led to the discovery of mutagenic agents specific for chloroplast genes (Gibor and Granick, 1964). The presence of DNA in isolated plastids was often reported in the literature (Chiba and Sugahara, 1957), but these results were always open to the objection that the DNA obtained arose from contamination by nuclear or bacterial particles.

Recent work has overcome these difficulties. Sager (1963) obtained a DNA from the chloroplast fraction of the green alga, Chlamydomonas. The chloroplasts when purified by centrifuging the isolated plastids on a discontinuous sucrose gradient yielded a chloroplast suspension freed from cell wall fragments, whole cells and whole nuclei. The DNAs

extracted from this purified fraction and those extracted from whole cells were subjected to cesium chloride gradient centrifugation. This technique enabled the investigator to separate different species of DNA with high degree of resolution and at the same time gave information about the base composition of the DNA species. The results of this gradient centrifugation indicated that two DNA species were present, a major one having a buoyant density of 1.728 and a minor band having a buoyant density of 1.702. The minor component is only 6 per cent of the total DNA in the extract of the whole cells, but makes up to 39 per cent of the total DNA isolated from the chloroplast fraction. The minor component was concentrated by procedures which purify the chloroplasts. Hence this minor component was considered to be chloroplast DNA.

Similar results were found using the cells of Euglena gracilis as chloroplast source (Brawerman and Eisenstadt, 1964). A method was developed by which purified chloroplasts were obtained; CsCl density centrifugation was used to achieve the final DNA separation. When a strain of Euglena which had lost the ability to form plastids was used to prepare DNA, the minor component was no longer present (Ray and Hanawatt, 1965).

The green alga, Acetabularia, is uniquely fitted for investigations of this kind. The nucleus of the plant can be removed by mechanical means leaving intact the

chloroplast complement. A DNA has been isolated from chloroplasts of enucleate Acetabularia. The DNA content of the chloroplast fraction is 10^{-16} g. per cell (Gibor and Izawa, 1963). Balthus and Brachet (1963) also prepared chloroplast DNA from enucleate Acetabularia cells grown under aseptic conditions. In this case the DNA isolated from the cells could not be due either to nuclear or bacterial contamination.

Chloroplasts isolated from the leaves of higher plants have been shown to contain DNA. Chun, Vaughan and Rich (1963), using a CsCl gradient, showed the presence of a specific chloroplast DNA isolated from spinach and beets. The amount of DNA in the chloroplasts was about 1 per cent of the total DNA in the cell. Kirk (1963) was able to separate nuclei from chloroplasts of broad bean in a discontinuous sucrose gradient. The DNA associated with the chloroplast fraction had a different base composition from that associated with the nuclear fraction. Shipp, Kieras and Haselkorn (1965) isolated DNA from tobacco leaf chloroplast. The DNA of the plastids hybridized to the nuclear DNA to the same extent as the completely unrelated E. coli DNA hybridized to it.

Autoradiographic techniques have also been used to show the presence of DNA in the plastid. Wollgiehn and Mothes (1964) showed that tritiated thymidine was incorporated

into the chloroplasts of Nicotiana rustica cuttings. If DNAase was added to the leaves before fixation, no silver grains appeared under the plastids. No such effect was observed with RNAase treatment. Plastids in older leaves incorporated significantly less radioactivity than did those in younger leaves. Tritiated thymidine has also been shown to be incorporated into the plastids of Spirogyra (Stocking and Gifford, 1959), and Euglena (Sagan, Ben-Shaul, Epstein and Schiff, 1965).

Electron microscopic evidence for DNA in the plastid has been reported by Ris and Plaut (1962) for plastids of Chlamydomonas. Similar pictures were taken of the chloroplast of Swiss Chard proplastids (Kislev, Swift and Bagorad, cited in Gibor and Granick, 1964).

MATERIAL AND METHODS

Plant material

Pisum sativum var. Alaska was used in most experiments. The plants were grown for nine to fourteen days in a growth chamber at 80°F under 14 hours of light and 10 hours of darkness. Dark grown plants were grown in another growth chamber at 70°F. In some of the early experiments, oats, mustard greens and peanuts were used as plant material. None of these plants were as satisfactory as pea in either ease of release of plastids from cell or length of time necessary to grow the plant to obtain a sufficient quantity of leaf material. The leaves and the apical tip of the plant were the only portions used as source of plastids.

Preparation of isolated chloroplasts

Cell breakage: Several methods were employed. In early experiments an omni-mixer was used. With oat leaves this instrument was necessary to obtain a high degree of cell rupture. The leaves were cut up with a razor blade and placed within the homogenizer together with the isolating medium. The varistat was set at 35 and homogenization proceeded for 3 minutes. A cell homogenizer, based on an adaptation of a standard glass homogenizer suggested by

Brendler (1951), was utilized to homogenize the leaves of pea, peanut and mustard green. The technique most commonly employed was breakage of the leaf material in a chilled mortar. Even though this technique was less effective than the Brendler technique in homogenization of leaves, it was thought to produce less deleterious effects on the isolated chloroplasts.

At the beginning of the homogenation procedure, the leaf material was added to the chilled mortar and only enough isolation medium to wet the leaves was added. More medium prevents the close contact between mortar and cells which is necessary for this breakage. Walker (1965) observed that the breakup of isolated chloroplasts in aqueous medium is inhibited by sucrose. One gram of solid sucrose was added to the leaves before grinding. No attempt was made to find out the effect of this on the isolated chloroplast. In some cases it was difficult to achieve a high degree of cell breakage. Several different abrasive substances were tried before adsorptive alumina was chosen.

Several kinds of isolating media were tried. Basically all were sucrose media to which various other substances were added because of their supposed ability to protect the plastid from disintegration. A medium suggested by Brawerman and Eisenstadt (1964) was used for the preparation of plastids. The medium consists of 0.25M sucrose, 0.004M MgCl_2 , and 0.001M mercaptoethanol in 0.01M Tris HCl pH 7.7. Another medium

used was one developed for the stabilization of isolated chloroplasts; the so-called W medium was made up of 0.25M sucrose, 25 per cent Ficoll (a polymer of sucrose obtained from Pharmacia, Fairlawn, N.J.), 5 per cent dextran, 0.006M mercaptoethanol, 1 per cent bovine serum albumin, and 0.001M each of $MgCl_2$, $MnCl_2$, and $CaCl_2$ in 0.01M Tris HCl pH 7.8.

Purification of plastids.

The broken material was filtered through two thicknesses of cheesecloth. Any alumina present was removed by a 5-minute centrifugation at 500 X g. Whole chloroplasts, and cell and wall fragments were sedimented by a 10-minute centrifugation at 3,000 X g. The 3,000 X g sediment was further purified by suspending the material in 15 ml. of a 2.0M sucrose solution and floating this on a 2.5M sucrose solution. This discontinuous gradient was centrifuged at 33,000 X g for 15 minutes in rotor 25.1 of a Spinco Model L preparative ultracentrifuge. Cell and wall fragments, starch grains and some plastids formed a precipitate at the bottom of the tube. Most chloroplasts accumulated at the interface between the two sucrose solutions. The top layer of sucrose solution, for the most part free of plastids, was removed by means of a syringe. More isolation medium was added to the top of the chloroplast layer, and the chloroplasts suspension was carefully removed from the bottom layer of sucrose by means of a syringe. Several

small portions of W medium were carefully added to facilitate chloroplast removal. A repetition of this procedure did not result in any appreciable further purification of plastids since little precipitate formed as a result.

The chloroplast suspension was diluted 1 x with cold isolation medium to ensure that the medium was not too dense to prevent the precipitation of the whole plastids. A second 3,000 X g centrifugation was performed. The sediment consisted chiefly of whole chloroplasts possessing bounding membranes. The supernatant consisted mostly of a small number of broken plastids which were usually discarded. The fraction sedimenting during the second 3,000 X g sedimentation is referred to as the chloroplast fraction. That fraction sedimenting through the 2.5M sucrose is referred to as the nuclear fraction.

The first 3,000 X g supernatant usually still contained a large amount of membrane-bound chlorophyll. A second 3,000 X g or a 10,000 or 20,000 X g sedimentation failed to removed this pigment and also failed to precipitate the entire amount of chlorophyll from the supernatant. Sedimentation at 30,000 X g, however, removed most of the pigment leaving a greenish or yellowish supernatant which contained mostly alkali labile uv absorbing material. The supernatant was usually discarded. The 30,000 X g sediment

which consisted chiefly of broken plastids with some mitochondria and nuclear debris is referred to as the broken plastid or mitochondrial fraction.

Extraction of Nucleic Acids

Preparation of undenatured nucleic acid from chloroplast and mitochondrial fractions

The fractions were either extracted with detergent and then extracted with phenol or were extracted with phenol alone. The chloroplast and mitochondrial fraction precipitates were suspended in cold 0.01M Tris, 0.06M KCl, 0.01M $MgCl_2$. A small aliquot was usually removed at this point for determination of chlorophyll content according to the procedure of Arnon (1949). An equal volume of cold phenol washed with Tris medium was added. The phenolic layer was mixed with the chloroplast suspension by stirring. The phenolic layer was separated from the aqueous layer by centrifugation at 23,000 X g for 10 minutes. The aqueous layer containing the nucleic acids was removed by means of a syringe. One phenol extraction was usually sufficient for complete removal of chlorophyll and the complete denaturation of the proteins. The nucleic acids were precipitated by 2 volumes of ice cold ethanol in which 20 mg. sodium acetate per ml of solution had been previously dissolved. The precipitate was suspended in buffer, usually

0.15M NaCl and 0.015M sodium citrate (saline-citrate). The solution was dialyzed for 48 hours in the cold against the nucleic acid solvent to remove an optically dense component with an absorption maximum of about 270 mμ. This component has a similar absorption spectra to phenol. In later experiments it was found that the contaminate could be eluted from a methylated albumin column by low ionic strength buffers such as 0.1M NaCl in 0.05M phosphate buffer pH 6.7. Neither the protein nor any absorbed nucleic acids appeared to be adversely affected by this procedure. All nucleic acid fractions were still elutable from the column when buffers of higher ionic strength were passed through it. The methylated albumin column was prepared according to the procedure of Mandell and Hershey (1960), with all materials doubled.

Extraction of nucleic acids from whole leaves and from bacteria

It was sometimes necessary to extract the nucleic acids from unbroken plant material or from a culture of bacteria. Phenol extraction was the method usually employed. The material to be extracted was placed in an omni-mixer vessel in a medium consisting of Tris medium added in concentration of 1 ml/g fresh weight of tissue, 1 ml of 40 mg/ml bentonite solution for every 10 g fresh weight of tissue and 1 ml of 2.2 per cent sodium lauryl sulphate per

10 g fresh weight of tissue. An equal volume of Tris washed phenol was added and the material homogenized at full speed for one minute. At that time the omni-mixer was slowed to low speed and the material homogenized at this speed for one minute. A final 1-minute extraction at full speed completed the breakup of the tissues. Less drastic procedures resulted in failure to release all the nucleic acids present. E. coli DNA could be effectively released by grinding the cells in a chilled mortar with alumina followed by phenol extraction of the homogenate. Several extractions with phenol were usually necessary to free the homogenate prepared from whole cells of protein.

Purification of the extracted nucleic acids

Separation of the DNA from the RNA: Since RNA has a similar absorption spectra to DNA, it was necessary to remove it if any estimation of the amount of DNA present was to be made by optical means. RNA was partially degraded by digestion with Bovine pancreatic RNAase (Sigma Ribonuclease fraction XIIA). An equal volume of the enzyme solution was added to the sample. The concentration of enzyme was 0.1 mg/ml in acetate buffer pH 6.0. The digestion was allowed to proceed for one hour at room temperature. The enzyme was removed by adding 1 ml of bentonite suspension (40 mg/ml) and centrifuging down the bentonite. The degraded RNA was separated from the DNA by absorbing it onto a methylated

albumin column and eluting the degraded RNA with 0.35M NaCl. in 0.5M phosphate buffer pH 6.7. The DNA not hydrolyzed by the enzymatic digestion remained on the column. It was eluted from the column by using buffered 1.0M NaCl.

Separation of contaminating nuclear DNA from chloroplastic DNA

Detergent extraction of chloroplasts: Brawerman and Eisenstadt (1964) found that it was possible to separate chloroplast DNA from nuclear DNA by extraction of the plastid suspension with a detergent, 5 per cent sodium deoxycholate. Chloroplasts were suspended in 100 ml 0.01M Tris buffer pH 6.7 containing 0.01M $MgCl_2$. Five ml of 5% sodium deoxycholate were added to the chloroplast suspension and the suspension was carefully mixed. Chloroplast fragments were precipitated by a 30 - minute 30,000 X g centrifugation. If sucrose was present in the medium to any degree, the plastid fragments tended to remain in suspension even after centrifugation at 30,000 X g. Sucrose was often carried over from earlier parts of the experiment. A preliminary washing with Tris was often necessary to remove this material.

Characterization of the DNA fractions isolated

Cesium chloride gradient centrifugation: Chloroplast DNA may be separated from other kinds of DNA by cesium chloride gradient centrifugation. Optical grade CsCl obtained from Alfa Inorganics, Beverly, Massachusetts, was made up in 0.02M Tris buffer pH 8.5. This stock solution of saturated CsCl which had a mass density of about 1.9239 g/cm³ was diluted to the required density with a solution of DNA in Tris buffer. The density of the CsCl solutions was determined by measuring the index of refraction of the solutions by means of a refractometer. A formula given by Vinograd and Hearst (1962) relating index of refraction to buoyant density was used to calculate these values.

At first, rotor 39.1 of the Spinco Model L preparative ultracentrifuge was used. The DNA containing CsCl solutions were adjusted to a density of 1.700. E. coli DNA was used as a marker and the solutions were centrifuged at 120,000 X g for three days at 18-19° C. Later an attempt was made to form a gradient by mixing solutions of CsCl of known densities. The gradient employed called for a 6 ml preformed gradient using 3 ml of a CsCl solution of density 1.800 and 3 ml of one of density 1.600. The low density solution was placed in the mixing chamber of a plexiglass gradient maker, the higher density one was placed in the other chamber. All connecting spaces were filled with liquid to prevent the formation of

air bubbles. A slight air pressure was applied to each chamber. The connecting valves were opened slightly to the same degree and the two liquids allowed to mix together with the magnetic stirrer operating at full speed. The hypodermic needle attached to the effluent opening was placed as near to the bottom of the centrifuge tube as possible without preventing the flow of the solution. Every precaution was taken to prevent the mixing of the preformed gradient during manipulations before centrifugation. Samples were placed in CsCl solution of density 1.600 and added to top of gradient.

According to Fisher, Cline and Anderson (1964) a fixed-angle rotor will also permit the establishment of a linear gradient within it which remains undisturbed by the reorientation of the tube contents when the sample is removed from the rotor. Since rotor 39.1 of Beckman Model L ultracentrifuge was not always available, rotor 50, a fixed-angle rotor, was used in its place.

The hemispherical portion of the centrifuge tube was filled with a dense liquid, usually a CsCl solution of density the same or greater than the most dense portion of the gradient. The portion of the tube unoccupied by liquid or cap was filled with mineral oil. The preformed gradient was centrifuged at 120,000 X g for 12-24 hours. The temperature of the internal chamber of the centrifuge was maintained close to 25°C.

After centrifugation the tubes were removed from the rotor and the caps were taken off. A hole was punched in

each tube using a tube punching apparatus adapted from a design by Salo (1965). A piece of rubber tubing exactly fitting the outside of the centrifuge tube, but permitting the tube to slip inside, was selected. A rubber stopper was fitted into one end of the tubing. A hypodermic needle was punched through the cork and the cork was held strongly by means of a clamp. A number 20 needle was used for this purpose. The sharp end of the needle was set in the correct place to collect the gradient without collecting the solution occupying the hemispherical portion of the tube. A soft rubber septum from a syringe bottle was used to seal the place where the needle penetrated the stopper to prevent the leakage of gradient material at these points. A hole had been pierced in the cork at a spot not touched by the bottom of the centrifuge tube to prevent mixing of the gradient.

Five drops were collected from the end of the needle in each collecting tube. The uv absorbance in each tube was determined on a Beckman DB spectrophotometer using a de-ionized water blank. The samples were read in a microcuvette having a critical volume of 0.3 ml and a light path of 0.1 cm.

Analytical Procedures

DNAase treatment: Sigma Bovine Pancreatic DNAase Fraction XII was made up in 0.001M MgCl_2 and 0.01M phosphate buffer pH 7.0 to a final concentration of 1 mg/ml. The volume of DNAase added to the sample was one-tenth of the sample volume. The solution was allowed to stand at room temperature for an hour and the enzyme was removed by precipitation with bentonite. This treatment provided sufficient depolymerization so that the DNA was eluted from a methylated albumin column with 0.35M NaCl instead of 1.0M NaCl.

Acid extraction and colorimetric determinations: In some cases it was not necessary to have undegraded DNA. Acid extraction procedures were used in those cases. The sample was treated with an equal volume of cold 1.0M HClO_4 . With large volumes a small volume of cold concentrated perchloric acid was used to bring the concentration of the acid in the sample to 0.5M. After centrifugation, the acid soluble portions were discarded and the precipitate was first washed with ethanol; then with a mixture of ether, chloroform, and ethanol (2:1:2) by volume to remove chlorophyll and lipids. RNA and DNA were solublized by hot acid extraction at 70-90°C for 15 minutes. RNA was sometimes removed before the hot acid hydrolysis by alkaline hydrolysis using an equal volume of 0.6N KOH for 18 hours at room temperature. Colori-

metric tests were made on the acid hydrolysate. The diphenylamine procedure of Burton (1956) was commonly used.

Hydrolysis of DNA: DNA was hydrolyzed by an enzymatic procedure in which the molecule was first attacked by pancreatic DNAase and then by phosphodiesterase Sigma type II isolated from snake venom. The enzyme was removed by treating the solution with 0.5M HClO₄ and filtering the material through a millipore filter paper. The solution was neutralized and its volume reduced in a boiling water bath. Nucleotides released were separated by thin layer chromatography, and the nucleotides eluted from the cellulose and estimated by uv absorption. These experiments were not completed. Acid hydrolysis of DNA was also tried; 0.5M acid was used to release the purines.

Thin layer chromatography

DEAE cellulose powder (MN cellulose powder 300 DEAE) was obtained from Brinkman Instruments, Great Neck, New York. The powder was washed with 500 ml of a mixture of 0.1M formic acid and 0.1M sodium formate. This washing was necessary to remove iron compounds which were heavy contaminants of the powders imparting to them a yellow color. The cellulose was spread on glass plates and dried. Nucleotide separation was best obtained with a solvent made up of saturated ammonium sulfate, 2.0M sodium acetate, and isopropanol (88:18:2).

P³² labelling experiments

The tops of pea plants were cut off with a razor blade at the lowest node and the cut end placed into deionized water containing the P³². Most often the amount of P³² in the solution was 50 uc in 25 ml of deionized water. The chloroplasts were prepared and extracted as described previously. Counting was done on a Nuclear Chicago gas flow counter in stainless steel planchets.

DNA melting point determinations

Isolated DNA was suspended in saline-citrate. The sample was heated in a cuvette within the chamber of a Beckman DU spectrophotometer fitted with thermospacers and insulators. The thermospacers contained tubes permitting the circulation of water from a Precision Scientific Circulating water bath controlled by a mercury thermoregulator. The phototube and the optical portions of the DU were protected from overheating by another set of thermospacers in which water at room temperature was circulated. A single cuvette next to the sample one was fitted with a thermocouple wire attached to a potentiometer calibrated in even numbered degrees Fahrenheit. Thus it was possible to read to the nearest whole degree Fahrenheit. It was possible to follow the increase in optical density of the DNA solution as the temperature was slowly increased. The procedure followed and the calculations used in determining the base composition of the sample were those of Marmur and Doty (1962).

RESULTS

The use of methylated albumin columns in the fractionation of nucleic acid extracts

In this work, methylated albumin columns were used extensively in the final stages of preparation of nucleic acids. The methylated albumin combines with nucleic acids. Increasing the ionic strength of the medium causes the release of the nucleic acid from the protein in a characteristic manner. Two methods were used to elute the nucleic acid from the protein. In the first method, a linear gradient elution with sodium chloride was used. Nucleic acids appear in a characteristic sequence from the column. In the second method, known volumes of specific concentrations of sodium chloride were added to the column. The different species of nucleic acids were eluted at different concentrations.

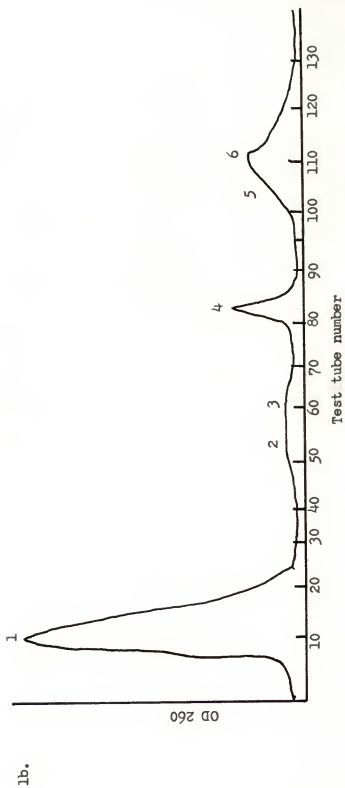
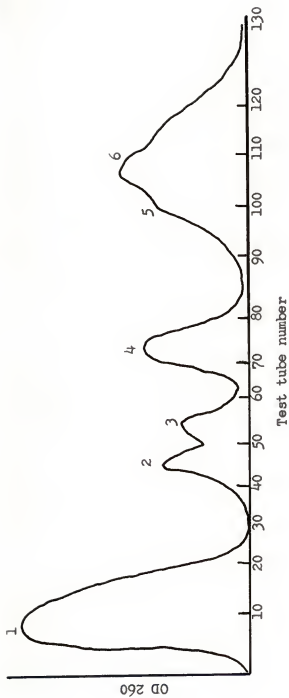
Figure 1 shows two uv absorption tracings of an eluate from the column passing through a cuvette and intercepting a uv light beam. The size and height of the peak arising from this procedure depends on a number of factors, such as flow rate of sample through cuvette, other than the amount of nucleic acid species present. This makes it unlikely that these will provide direct information about the re-

Fig. 1a.-A uv absorbance tracing from the eluate of a methylated albumin column of a nucleic acid fraction prepared by phenol extraction of 10 grams of oat leaves grown in light.

An omni-mixer was used to release the nucleic acid from the cells. One-half of the sample was added to the column without dialysis. The sodium chloride gradient was 400 ml 0.3M NaCl - 400 ml 1.3M NaCl.

Fig. 1b.-A uv absorbance tracing from the eluate of a methylated albumin column of a nucleic acid extract prepared using a Brendler teflon pestle homogenizer to break up the cells of oat leaves grown in the light.

Phenol was used to denature proteins and remove lipid. One-half of the sample was placed on the column and the material eluted by using a linear NaCl gradient of 0.3 - 1.3M NaCl; 400 mls of each solution were added to the gradient maker.



amounts of nucleic acid in each fraction. Rather, it was necessary to read the amount of absorbancy at 260 m μ of each fraction on a Beckman DB spectrophotometer to be certain of this. In most experiments this was not done.

The sequence in which the nucleic acids species are eluted from the column was usually the best diagnostic test for their identity. Figure 1a shows a tracing of an eluate from a methylated albumin column eluted by a linear salt gradient. The column was loaded with half of a sample of nucleic acids prepared from 10 grams of oat leaves. Six peaks are evident. The first peak is phenol. Its absorption maximum is at 270 m μ . All other peaks showed absorption maxima at 260 m μ or slightly below. These peaks were sensitive to RNAase. Either the peak completely disappeared or lost a considerable amount of uv absorbing material when sample was treated with the enzyme. The uv absorbing material usually appeared in the first soluble RNA peak. (See Figure 5). Peak 4 was always partially resistant to RNAase treatment. DNAase treatment, however, caused the peak to completely disappear. The uv absorbing material from this peak eluted within the first twenty fractions collected. This is true for all peaks whose material was sensitive to the enzyme. No other peaks were sensitive to DNAase. Thus, peak 4 was believed to be made up of a DNA/RNA complex of non-specific nature. All other peaks are entirely due to different RNA species.

Peaks 2 and 3 usually occur in such small amounts that it is difficult to tell if they are present or not. The large amount of nucleic acid that is present in this sample was the reason that so much uv absorbance appeared in these fractions. These two peaks are probably due to soluble RNAs of the transfer variety. The last two peaks, 5 and 6, are due to ribosomal RNA usually present in the highest concentration of all nucleic acids in the samples. The base composition of the RNA in these peaks resembles that reported for ribosomal RNA in the literature. These two peaks usually overlap although the boundary between them is somewhat hard to ascertain in many tracings.

This sequence of nucleic acid elution is not changed by many factors which change the shape and size of the peak. Factors such as flow rate and steepness of the salt gradient used as eluting agent, can change the size and shape of the peak as well as the fraction into which the peak is eluted, but do not change the sequence which nucleic acid species are eluted. This is indicated by comparing Figure 1b and Figure 1a. The same sequence of elution is shown by samples prepared by two different procedures. The sample shown in Figure 1b was prepared by using a Brendler cell homogenizer. This treatment does not release as many of the cellular nucleic acids as the omni-mixer used to prepare sample in Figure 1a. Yet, the two-humped ribosomal RNA peaks appear near the end of

the gradient. Peak 4, the DNA/RNA peak was eluted within ten tubes of the ribosomal RNAs. The soluble RNA peaks are so small that they are difficult to see on the tracing from the column.

Fractionation of nucleic acids isolated from the chloroplast fraction

When nucleic acids extracts from chloroplast samples were placed on a methylated albumin column the results were equivocal. Figure 2 shows a tracing from a chloroplast fraction isolated from oat leaves. One peak of uv absorbance is present. This peak has an absorption maximum at 260 mu. Color tests were not performed because the material was used up in running CsCl gradients. The absence of other peaks in the eluate made identification of the peak difficult. This was the first good evidence for the presence of nucleic acid in the chloroplast fraction found in this project.

The tracing obtained from phenol extracted chloroplasts from dark grown oat leaves exposed briefly to light gave more definitive results. These are shown in Figure 3. Since optical density readings of the contents of most of the test tubes were taken, more meaningful information is conveyed by this figure than was possible with the others. Three peaks appeared. The third peak was probably due to ribosomal RNA. These ribosomes came from the chloroplasts since a peak at this position was never found in tracings of

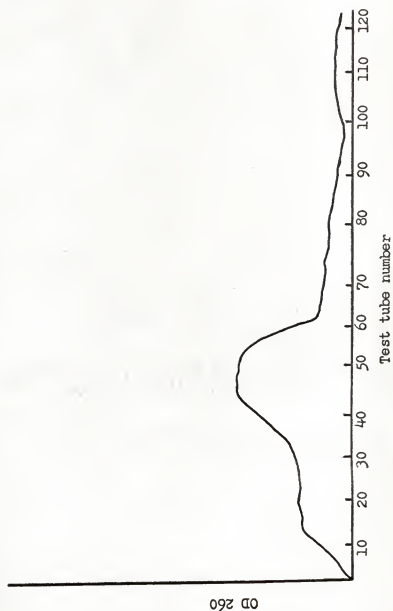
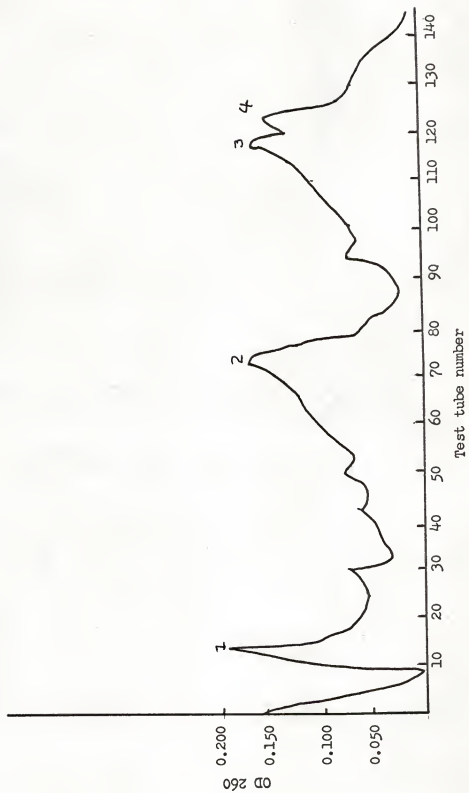


Fig. 2.-A uv absorption tracing from a methylated albumin column of nucleic acid prepared from the chloroplast fraction of oat leaves.

Fig. 3.-The distribution of uv absorbance among fractions collected from a methylated albumin column.

Dark grown oat plants were exposed briefly to light and the chloroplast fractions isolated and extracted with phenol.

A linear sodium chloride gradient of 400 ml. 0.3M NaCl - 400 ml. 1.3M NaCl was employed as the eluting agent.



extracts made from chloroplasts of light grown leaves as would be expected if microsomal ribosomes were contributing to this peak. (Figures 2 and 4). A high RNA content was also indicated by results found with acid fractionation of chloroplast fractions using the procedure of Ogur and Rosen (1950). Cold extraction of acid insoluble material for 18 hours with 1 M perchloric acid supposedly results in the solution of RNA, leaving any DNA present in the residue. The cold acid extract of a chloroplast fraction from dark grown plants exhibits greater uv absorbance than that of light grown plants (see Table 1). Smillie, Evans, and Lyman (1963) found that dark grown Euglena chloroplasts had higher RNA content than those exposed to light for periods greater than 12 hours. After this time the RNA content fell to a level markedly below the 12 hour level. Peak 2 is probably a DNA peak but corroborative evidence is lacking.

Figure 4 shows a tracing obtained from the chloroplasts of light grown mustard green leaves. The second uv peak is in the approximate position of the second peak of Figure 3. No other tests were run on this sample.

The distribution of nucleic acids among fractions prepared during the isolation of chloroplasts

The distribution of DNA in the different fractions prepared during the isolation of chloroplasts from pea leaves was studied by means of their uv absorbance spectra

TABLE 1

THE DISTRIBUTION OF 260 mμ ABSORBANCE IN COLD ACID EXTRACTS OF CHLOROPLAST SAMPLES^a
 PREPARED FROM 20 GRAM SAMPLES OF OLD LEAVES; ONE GROUP OF PLANTS WAS GROWN FOR
 THREE DAYS IN THE LIGHT, THE OTHER WAS GROWN IN THE DARK

Fraction	18 hours extraction				1 hour extraction				OD 260				1st washing				2nd washing				Protein mg/sample				Chloro- phyll mg/sample			
	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light	dark	light
Chloroplast	2.0	0.37 ^b	0.33 ^b	0.10 ^c	0.04	0.20	0.00	0.08 ^c	7.96	7.90	626																	
Low density chloroplast	1.6	0.22 ^b	0.12 ^b	0.17 ^c	0.037 ^b	0.08 ^c	0.09	0.00	2.60	5.18	324																	
Mitochondria	1.4	2.0	0.125 ^b	0.457 ^b	0.027 ^b	0.22	-	0.19	9.78	5.84	603																	
Total in fraction									20.34	18.92																		

^aThe leaves were homogenized for three minutes in 0.5M sucrose containing 0.01M Tris, pH 7.8 and 0.01M CaCl₂ in an omni-mixer at 35.

The chloroplast fraction was prepared by sedimenting the homogenate at 2,000 X g for 15 minutes. The supernatant from this step was used to prepare the low density chloroplast fraction by sedimentation at 4,500 X g for 15 minutes. The mitochondrial fraction was prepared by sedimentation at 24,400 X g for 15 minutes.

The above fractions were treated with 1M perchloric acid in cold. The acid precipitate was separated by sedimentation and washed with boiling ethanol and with

a (cont'd)

a mixture of ether, ethanol and chloroform (2:2:1). The samples were treated with 5 ml. of cold perchloric acid for 18.5-hours. The supernatant was saved and its optical density read on a DB spectrophotometer. An additional 5 ml. of cold perchloric acid was added and the extraction was continued for one-hour. The precipitate was then washed twice with 5 ml. of 1*M* perchloric acid. Substances soluble in hot acid were removed with two extractions in 0.5*N* HClO_4 for 15-minutes. The residue was used to perform protein tests according to the method of Lowry (1957). Chlorophyll was determined by the method of Arnon (1949).

^b Absorption maximum at 260 mu.

^c Absorption maximum not at 260 mu.

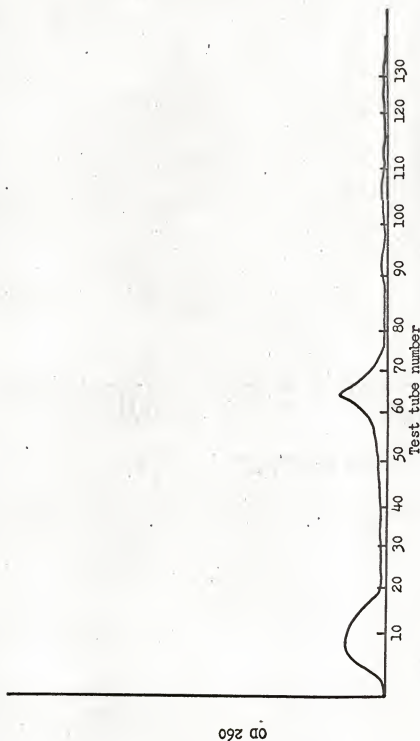


Fig. 4.-A uv absorbance tracing from the eluate of a methylated albumin column of a nucleic acid fraction isolated from the chloroplast fraction of mustard green leaves prepared by phenol extraction. The sodium chloride gradient used for elution was $0.3M$ - $1.3M$ NaCl; 400 ml of each were employed.

and the spectra of the blue colored substance formed by the reaction of diphenylamine with deoxyribose. Tables 2 and 3. The results obtained were not consistent because of the low sensitivity of the diphenylamine (DPA) reagent to low concentrations of DNA. Larger amounts of material or more sensitive methods such as those in the next section were needed.

Detergent extraction of chloroplasts

Chloroplasts contain two types of membrane systems: an internal one and an external one. Evidence summarized in the discussion section suggested that the chloroplast DNA is bound in some manner to the internal membranes which are not destroyed by detergent treatment. The external membranes are ruptured by the treatment. Nuclear membranes are known to be more fragile than chloroplast internal membranes. It is extremely difficult to prepare whole nuclei from plant cells using the techniques employed to isolate chloroplasts. No free nuclei were ever demonstrated in these experiments in preparations stained by the acetocarmine, acetoorcein or Fuelgen procedures. Detergent treatment should provide a means of solubilizing the nuclear fragments associated with the chloroplast fraction while retaining the chloroplast DNA inside the lamella system which sediments at 30,000 X g.

Table 4 showed evidence that a uv absorbing material was released from the plastids by detergent treatment. DPA tests were negative. The sample may have been too small

TABLE 2

THE DISTRIBUTION OF NUCLEIC ACIDS ISOLATED FROM EIGHT GRAMS OF PEA LEAVES IN FRACTIONS PREPARED DURING THE ISOLATION OF CHLOROPLASTS

Fraction	Preparation	OD260	Dilution	Maximum absorbance point	OD600	Maximum absorbance point
Chloroplast precipitate	3,000 X g precipitate position in sucrose gradient: in between the layers	0.78	1:10	260	0.11	600
Chloroplast supernatant	3,000 X g precipitate remained suspended in top layer of sucrose gradient	0.44	1:10	261	0.045	600
Mito-chondrial precipitate	25,000 X g precipitate	0.54	1:10	260	0.059	above 620
Mito-chondrial supernatant	25,000 X g supernatant	1.60	1:20	259	0.025	above 610

TABLE 3

THE DISTRIBUTION OF NUCLEIC ACIDS AMONG FRACTIONS* PREPARED DURING CHLOROPLAST ISOLATION FROM TEN GRAMS OF PEA LEAVES

Fraction	OD260	Dilution	Maximum absorption point	OD600
1	1.2	1:1	260	0.00
2	0.79	1:1	260-270	0.00
3	1.60	1:1	260	0.00
Chloroplast precipitate	0.791	0	270	0.00

270 mu maximum probably phenol

* The leaves were ground in W medium in a mortar. Chloroplasts in the homogenate were recovered by placing the extract over 10 ml of 1.5M sucrose and centrifuging at 32,449 X g for 15 minutes. Fraction 1 was the material remaining in the W medium, the first layer of the discontinuous sucrose gradient. The chloroplasts were re-suspended in W medium after removal from discontinuous gradient by means of a syringe. The chloroplasts formed a layer between the two layers of sucrose solutions. The re-suspended chloroplasts were re-precipitated by centrifugation at 30,000 X g. The supernatant was used to prepare fraction 2. Fraction 3 was prepared from the 1.5M sucrose layer. The chloroplast fraction was extracted with phenol and a part of it used in the color test. Each fraction was extracted with alcohol and acid and nucleic acids were solubilized by hot acid treatment.

or the DNA degraded to such an extent that little was left. Tables 5 and 6 show that DNA is present in the detergent supernatant fraction. Brawerman and Eisenstadt (1964) reported that a DNA present in the detergent supernatant fraction was recoverable by centrifugation at 100,000 X g for two hours. This procedure was repeated using the pea material. No DNA was detected by DPA activity, uv absorbtion or methylated albumin chromatography using a buffer system which had been successful in separating the different nucleic acid fractions from corn and pea samples.

The presence of nucleic acids in the detergent precipitates was much more difficult to show. The samples recorded in Tables 5 and 6 gave inconclusive DPA tests. A larger sample was needed. The results of using a larger sample are recorded in Table 7. Clearly both the detergent precipitate and supernatant fractions contain DNA.

The above tests are somewhat equivocal. The method used is somewhat insensitive and it is difficult to demonstrate the presence of DNA in every sample run. For this reason the more sensitive methods reported in the next section were developed.

The demonstration of the presence of DNA in extracts of chloroplast-containing fractions by means of nuclease treatments and methylated albumin column chromatography

Unequivocal demonstrations of the presence of DNA in fractions of isolated chloroplasts were very difficult. The DNA of the chloroplast was in such small amounts that it was measured by ordinary colorimetric determinations with difficulty.

TABLE 4

THE UV ABSORBANCY AND DPA ACTIVITY OF SAMPLES PREPARED BY DETERGENT EXTRACTION OF FRACTIONS PREPARED FROM LEAVES OF FLOWERING PEAS*

Fraction	Volume mls	OD260	Point maximum absorption	OD600	Point maximum absorption
Chloroplast precipitate	lost				
Mitochondrial precipitate	5	0.08	264	0.08	600
Chloroplast supernatant	2	0.34	252	0.01	none
Mitochondrial supernatant	5	0.18	260	0.00	none

*Eight grams of leaves were taken from flowering pea plants. Chloroplast and mitochondrial fractions were collected. After the second 3,000 X g precipitation of the chloroplast fraction from W medium, the chloroplast and mitochondrial fractions were made up to 10 ml with 0.01M Tris containing 0.01M Mg ions and 0.5 ml of 5 per cent sodium deoxycholate was added. The suspension was centrifuged at 30,000 X g for one-half hour. The detergent supernatants and detergent precipitates of both fractions were precipitated with acid. The uv absorbances and the intensity of color found upon addition of DPA reagent were measured.

TABLE 5

THE uv ABSORBANCE AND DPA ACTIVITY OF DETERGENT SUPERNATANTS
PREPARED FROM TURNIP GREENS LEAVES*

Fraction	OD260	Point of maximum absorbance	OD600	Dilution
Nucleus	0.78	260-264	0.115	1:4
Chloroplast	0.76	264-266	0.105	0

*Chloroplasts were prepared from the meristematic region and the youngest leaves of field grown turnip greens. A large amount of material was collected and immediately frozen in acetone/dry ice. The detergent precipitates of two runs were combined and saved for further study. The chloroplast fraction contained not only the material between the two layers but also that suspended in the upper layer. The plastids were suspended in 160 ml of 0.01M Tris pH 7.7 and 0.01M Mg ions. Seventeen ml of sodium deoxycholate were used. The detergent supernatant was adjusted to 0.5N HClO₄. The acid precipitates were washed with alcohol and solubilized by heating at 70° C for 15 minutes. The detergent precipitates were extracted with phenol and saved for further work.

TABLE 6

THE uv ABSORBANCES AND DPA ACTIVITIES OF DETERGENT SUPERNATANTS PREPARED FROM PEA CHLOROPLASTS***

Fraction	OD260	Point of maximum absorbance	Dilution	Volume of sample ml.	OD600	Volume of sample ml.**	Dilution	Point of maximum absorbance
Chloroplasts	0.66	258-264	1:4	3	0.23	1	0	590
Nucleus	0.64	260-268	1:9	3	0.21	1	1:4	590
30,000 X g* supernatant	0.78	262	1:99	4	0.09	2	0	none

*Not extracted with detergent.

**One ml. of each sample taken for DPA test.

***Pea leaves were also harvested. Twenty-two grams of leaves were ground in a mortar using alumina to break up cells. The 3,000 X g centrifugation was omitted. The 30,000 X g precipitate was purified on a discontinuous sucrose gradient in which the top layer was a 0.5M sucrose solution. The chloroplast fraction was that suspended in the top layer of the gradient as well as that at the interface between layers. The nuclear fraction was the precipitate at the bottom of the tube. The detergent extraction was done in the same way as with the turnip greens. Supernatants and precipitates were treated in the same manner.

TABLE 7

THE 600 μ ABSORBANCE OF DPA TREATED PHENOL EXTRACT OF DETERGENT PRECIPITATED CHLOROPLASTS ISOLATED FROM 21 GRAMS OF PEA LEAVES*

Fraction	OD 600	Maximum Absorption Point
Detergent precipitate	0.18	590-600
Detergent supernatant	0.15	Approximately 590

*Twenty-one grams of pea leaves were pulverized in a mortar. A small amount of alumina was added and 16.9 grams of solid sucrose was added to the W medium. The homogenate was centrifuged at 30,000 X g. The precipitate was suspended above 2.5M sucrose and centrifuged 15 minutes in rotor 25.1 at 32,449 X g in a model L preparative ultracentrifuge. The chloroplasts were removed from the interface between the solutions and re-precipitated by centrifuging at 30,000 X g for 15 minutes. Detergent treatment as described previously was carried out. An equal volume of chloroform, isoamyl alcohol (24:1) by volume was added to the detergent supernatant and the supernatant was stirred vigorously with a magnetic stirrer for one-half hour at room temperature. This procedure was adapted from a method of Marmur (1961), and was more convenient for large volumes of liquid, since the chloroform does not absorb at 260 μ . The two liquids are separated easily in a separatory funnel.

The detergent precipitate was extracted with phenol. Protein was removed from the aqueous phase to prevent its reaction with the DPA reagent interfering with the determinations. Each sample was treated with acid and the acid precipitate defatted with absolute ethanol. The precipitate was treated with 1 ml portions of 0.5N HClO₄ at 70°-85° C. The acid extract was filtered through a No. 1 filter paper and treated with diphenylamine reagent for 18 hours at room temperature. The samples were read in a Beckman DB spectrophotometer where the DPA blank in the sample position was set to zero against a de-ionized water blank.

The p^{32} labeling coupled with use of a purified DNA as a marker and the selective washing of methylated albumin columns overcame many of these difficulties.

In the first experiment of this series, nucleic acids were prepared by phenol extraction from the chloroplast and mitochondrial fractions of rooted peanut cuttings. The extract was treated with RNAase and the degraded RNA removed from the DNA by methylated albumin chromatography using a linear sodium chloride gradient. Phenol and other impurities were washed off the column using a low ionic strength buffer before the gradient was turned on.

The place where the DNA was eluted was located by using a sample of phenol extracted corn nucleic acids freed from the RNA components by the above procedure. Figure 5 shows an uv absorbance tracing of the marker corn sample before and after RNAase treatment. Test tubes 60 - 80 of the later sample were used as the marker.

The test tubes in the vicinity of the DNA marker eluted from the methylated albumin column along with the peanut sample were chosen for counting. Uv absorbance was read on a Beckman DB spectrophotometer. Acid precipitable material was collected on a Millipore filter paper and counted on a Nuclear-Chicago gas flow counter.

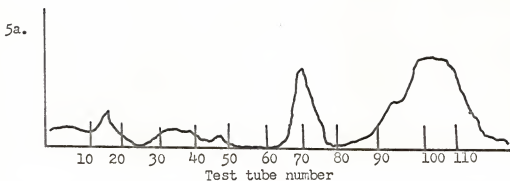


Fig. 5a.-A uv absorbance tracing of an eluate from a methylated albumin column. The sample was phenol extracted corn stem tips. The sample was untreated with RNAase. The DNA peak was used as a marker in experiments utilizing the chloroplast fraction from pea leaves.

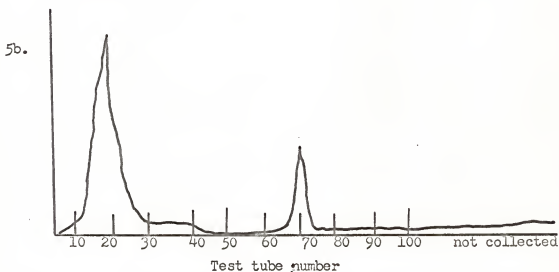


Fig. 5b.-A uv absorbance tracing of the other half of the corn sample shown in 5a. The sample was extracted with RNAase for one hour at room temperature.

The results of this experiment, shown in Figure 6, indicated that P^{32} was incorporated into the DNAs of the peanut chloroplast fraction. The amount of incorporation is very slight, however. Using the gradient it was found that the P^{32} peaks and the uv absorbance due to corn DNA overlap. It is not known whether the peaks would correspond better if pea nucleic acid were used instead.

This experiment was repeated using pea plants as the nucleic acid source. The nucleic acids were prepared, purified and counted using the same procedure as in the previous experiment except that fifty ml. aliquots of known concentrations of salt were used to elute the sample from the methylated albumin column. Degraded RNAs were first removed by washing the methylated albumin column with $0.35M$ NaCl. DNA was eluted by the second 50 ml aliquot of $1.0M$ NaCl. $0.35M$ salt did not elute it. Lower concentrations of salt did not elute the DNA. Results of this experiment are shown in Figure 7. The peak in radioactivity did not correspond to the peak in uv absorbance due to the corn DNA marker. However, the radioactive peak was eluted in the same position as the absorbance peak in most of the experiments. The pattern of uv absorbance elution found in this experiment is greatly different from that found in all other experiments. Most often an exact correspondence between P^{32} counts and uv absorbance was found.

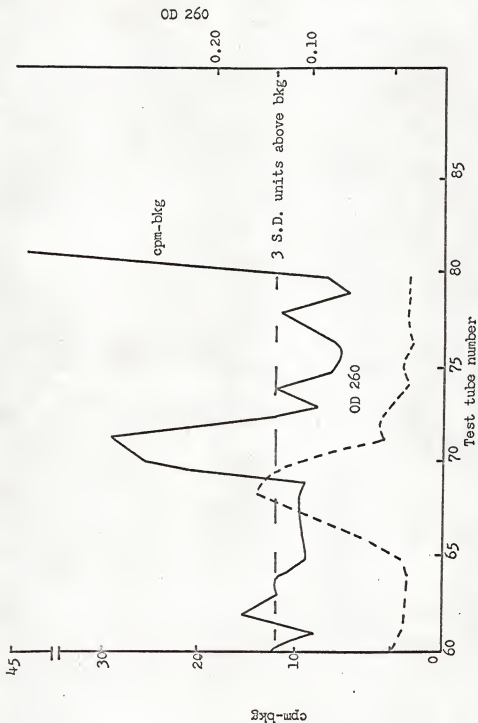


Fig. 6.-The incorporation of P^{32} into an RNAase resistant nucleic acid isolated from the chloroplast fraction prepared from rootless peanut cuttings. The sample was labeled with 50 MC P^{32} for 24 hours in light.

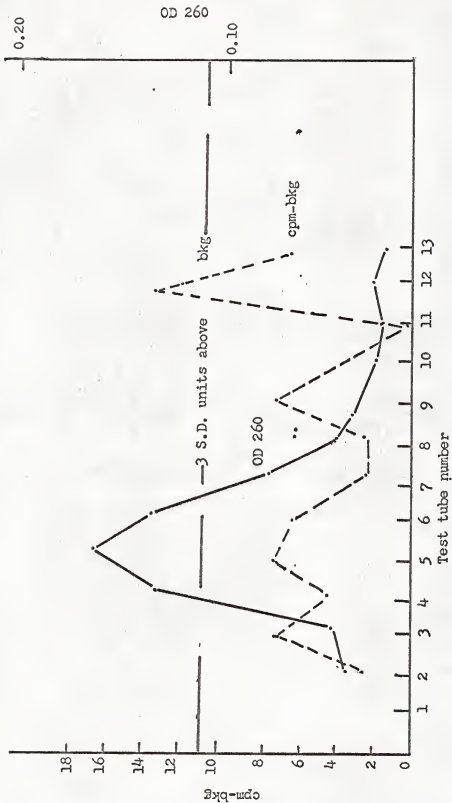


Fig. 7.-Incorporation of p^{32} into an RNAase resistant nucleic acid from the chloroplast fraction of pea tops excised from their roots. 50 light grown seedlings tops were labeled with 50 MC p^{32} for 24 hours. The samples collected are the second 50 ml aliquot of 1.0M NaCl previously washed with two 50 ml aliquots of 0.35M NaCl.

A third experiment was performed to see if excision of pea roots affected the results. Two batches of light-grown seedlings were selected. One batch had its roots excised, the other was left intact. Both were labeled with 50 μ C P^{32} for 24 hours. Nucleic acids were prepared, purified and counted as before. 1.2M NaCl was used to elute the DNA in this case only. The results of these experiments are recorded in Figures 8, 9, and 10. The DNA uv absorbance is again due to corn DNA. The absorbance and the count peaks coincide. The excised material apparently accumulated more counts in the chloroplast fraction's nucleic acids than the rooted plants did. This result is based on one experiment. It was decided to remove the roots in all the following experiments because no definite harm seemed to have occurred to the plants by such excisions.

Another group of experiments was undertaken in which the chloroplast and mitochondrial fractions were extracted with 5 per cent deoxycholate. As shown in Figure 11 the detergent precipitate shows a P^{32} count distribution similar to that found in previous work. The uv absorbance peak corresponds with the P^{32} count peak. Figure 12 reveals the fact that the 100,000 X g precipitate from the detergent supernatant fraction shows only doubtful incorporation of P^{32} .

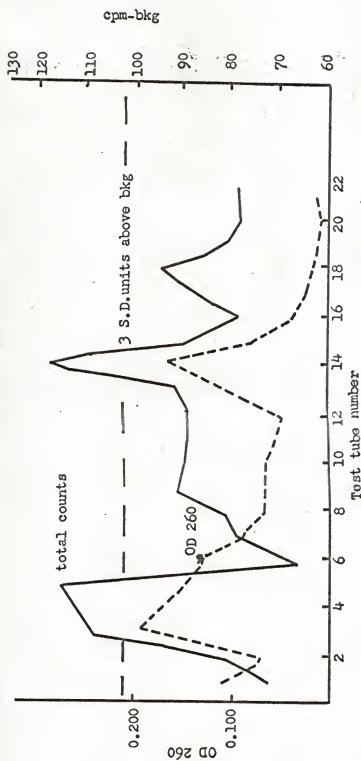


Fig. 8.-Incorporation of p^{32} into a RNAase resistant nucleic acid isolated from the chloroplast fraction prepared from pea plants with intact roots.

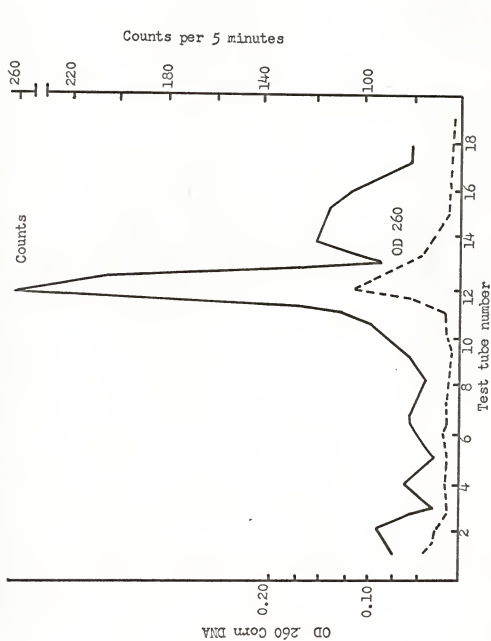


Fig. 9.—The incorporation of p^{32} into a RNAase resistant nucleic acid isolated from the chloroplast fraction prepared from leaves of pea plants excised from roots.

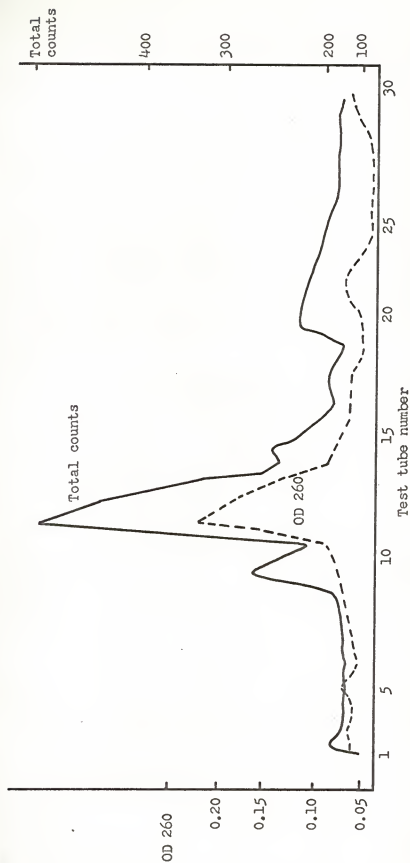


Fig. 10.-The incorporation of p^{32} into an RNAse resistant nucleic acid isolated from the mitochondrial fraction of leaves prepared from excised pea tops.

Fig. 11.-Incorporation of p^{32} into a RNAase resistant nucleic acid isolated from 30,000 X g detergent precipitate prepared from chloroplast fraction on pea leaves obtained from excised pea tops.

Twenty pea plants grown in dark for two weeks were excised from roots at lowest node and labeled for 24 hours with 50 μ c p^{32} dissolved in 50 ml of nutrient solution. The chloroplast and mitochondrial fractions were prepared and suspended in 100 ml of 0.01M Tris pH 7.7. The solution was made 0.01M in $MgCl_2$. Ten ml of 5 per cent sodium deoxycholate was added to the suspension, and the suspension was centrifuged at 30,000 X g for 30 minutes. The detergent precipitate was extracted with phenol and treated with RNAase for one hour at room temperature. The sample was absorbed on a methylated albumin column and the RNA and phenol washed off the column with 0.35M NaCl. The 1.0M NaCl eluted was collected and prepared for counting as related before. The samples were counted in a Nuclear-Chicago gas flow counter. UV absorption due to corn DNA was used as marker.

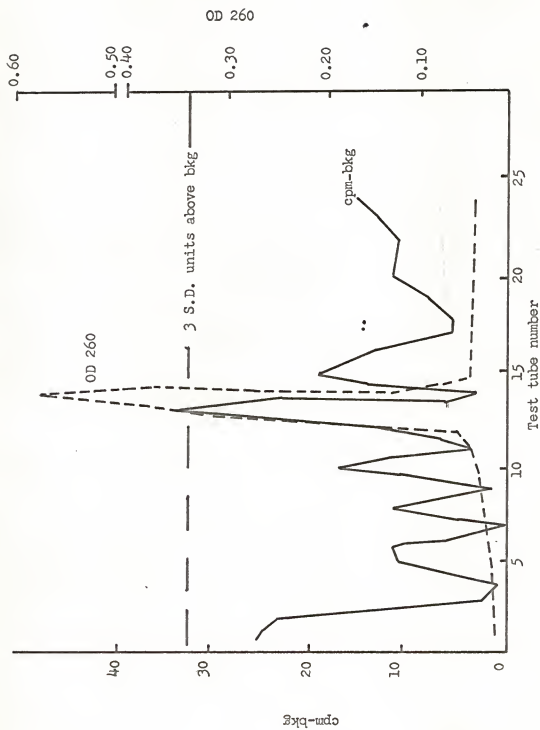
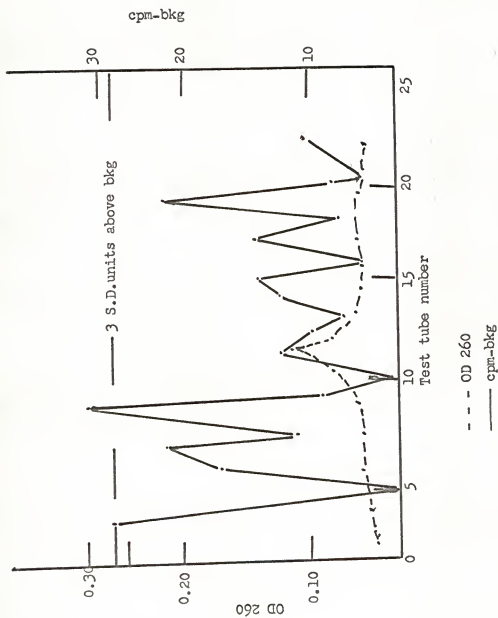


Fig. 12.-The incorporation of P^{32} into RNAase resistant nucleic acid isolated from the 100,000 X g precipitate prepared from the detergent supernatant fraction obtained from the chloroplast fraction of pea leaves from excised tops.

The detergent supernatant was centrifuged at 100,000 X g for two hours in a Spinco Model L Ultracentrifuge. The supernatant portion of this was discarded and the precipitate treated in the same manner as the detergent precipitate from the chloroplast fraction. Uv absorption due to corn DNA was used as a marker.



No coincidence of uv absorption maximum and P^{32} count maximum was observed in this case. This is in keeping with results found previously with colorimetric determinations. The results differ from those results obtained by Brawerman and Eisenstadt (1964), in which they found a DNA in the 100,000 X g precipitate prepared from the supernatant fraction of detergent treated Euglena chloroplasts.

Previous work with diphenylamine reagent seemed to indicate that the detergent supernatant fraction contained a DNA which was still present in solution after decantation from the 100,000 X g precipitate. This solution was discarded in the last experiment. These new experiments were designed to check on the presence of DNA in the detergent supernatant fractions. Figures 13 and 14 show that both the chloroplast and mitochondrial detergent supernatant fractions contain DNA. In this case more DNA seems to have been released from the mitochondrial fraction.

In interpreting these experiments, it becomes necessary to show that all of the RNA was degraded by the RNAase treatments. The philosophy behind these experiments is that when nucleic acid is prepared from whole leaves rather than from chloroplast fractions more RNA and DNA are released. If the same amount of enzyme is brought into contact with the larger sample and completely hydrolyzes it at room

Fig. 13.-The incorporation of p^{32} into an RNAase resistant nucleic acid isolated from the supernatant of detergent extracted chloroplast fraction prepared from leaves of excised pea shoots.

Twenty pea seedlings were excised at the lowest node and divided into two groups of 10 each, and labeled with 50 uc p^{32} in 25 ml deionized water. The labeling time was 24 hours of which 14 was under illumination of cool white fluorescent light bulbs supplemented with incandescent light. The chloroplast and mitochondrial fractions were prepared and extracted with detergent as in the last experiment. The detergent precipitates and the supernatant fraction resulting from detergent extraction were treated with phenol. The nucleic acids were precipitated with ethanol and the residue was digested with RNAase. The enzyme was removed by precipitation with bentonite and the nucleic acid fraction passed through a methylated albumin column which was washed with 0.35M and 1.0M NaCl to elute the nucleic acids. The 1.0M washings were collected and the uv absorbance due to added corn DNA read on a Beckman DB spectrophotometer. Nucleic acids present in the eluates were precipitated with cold perchloric acid and counted on millipore filter paper in a Nuclear-Chicago gas flow counter. Corn DNA was used as a marker.

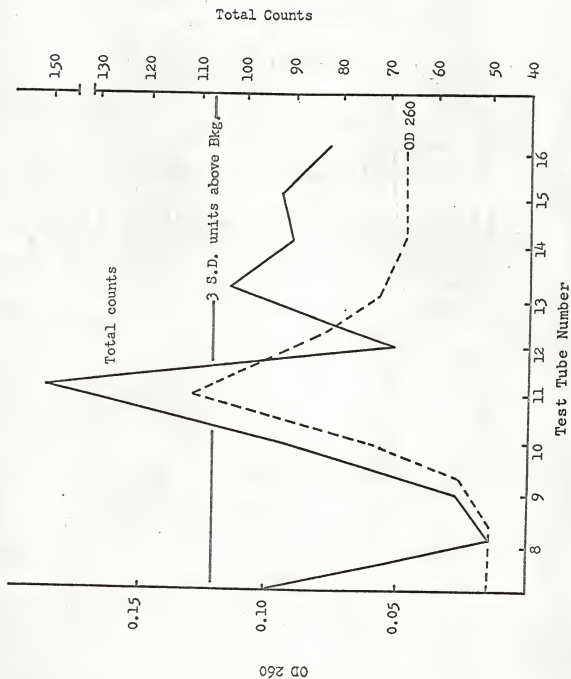
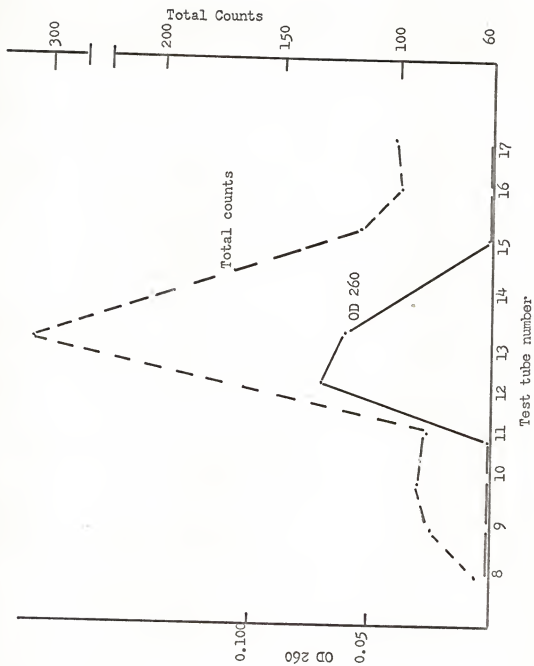


Fig. 14. The incorporation of p^{32} into an RNAase resistant nucleic acid isolated from the supernatant fraction of the detergent extracted mitochondrial from leaves of excised pea shoots.

(See Figure 13 for procedure used.) Corn DNA was used as a marker.



temperature with the same period of exposure which was used for the chloroplast extracts then this digestion procedure is more than adequate to be used in the purification of chloroplast DNA.

Deoxyribosenucleic acid was prepared from whole pea leaves by grinding the leaves in a mortar and pestle with phenol. Large unbroken fragments were re-ground using the same procedure. Deoxyribosenucleic acid was released by this process since long stringy fibers characteristic of the substance were observed upon precipitation with ethanol. The nucleic acids were taken up in 25 ml of saline-citrate and three 5 ml aliquots were taken for tests. In all cases, as shown in Figure 15, the amount of nucleic acids released was quite small; more probably remained present in the undegraded leaf material which was discarded.

To overcome this problem it was decided to use an omni-mixer to facilitate the breakup of the leaves. The nucleic acid suspension was divided into three portions of 3 ml each. One portion was treated for one hour with 100 ug/ml RNAase at room temperature. Another sample was treated with 0.3 ml of 1 mg/ml DNAase for one hour at room temperature. One portion was not treated with nucleases.

Nucleases were removed with bentonite. Each sample was fractionated by passing it through a methylated albumin column using a linear sodium chloride gradient in which 400 ml

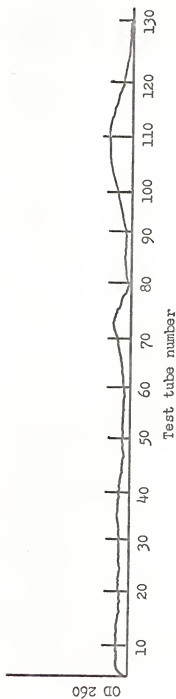


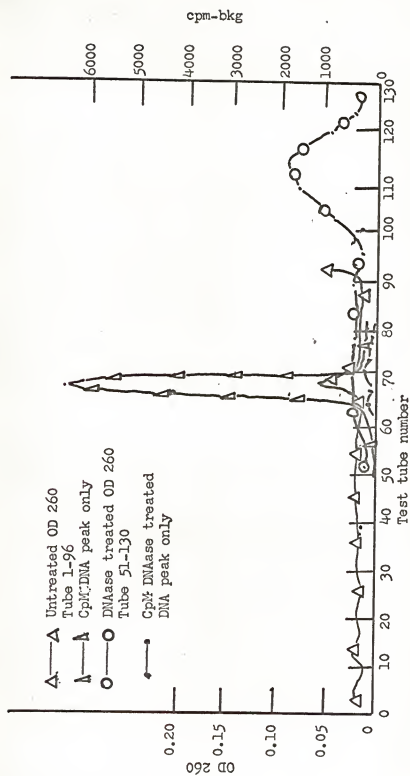
Fig. 15.-A uv absorbance tracing from a methylated albumin column of a sample of nucleic acids prepared from pea leaves by phenol extraction using a mortar and pestle to break up the pea leaf cells. The sample was prepared from leaves of 20 light grown pea seedlings labeled 24 hours with 50 μ c P₃₂ in 25 ml de-ionized water. The methylated albumin column was washed with a linear sodium chloride gradient ranging from 0.35M sodium chloride to 1.3M sodium chloride.

0.35M NaCl was added to the mixing chamber and 1.3M NaCl was added to the other chamber. The eluate was collected and the test tubes containing the peaks of interest were separated from the others. After reading the contents of the tubes on a Beckman DB spectrophotometer, two ml samples were removed, dried thoroughly, and counted. (Figure 16, 17). Clearly the two nucleases are effective at the concentrations employed in reducing the concentrations of the particular nucleic acid concerned. RNAase effectively reduces the level of counts in the ribosomal RNA peak that no significant number of counts appears at the part of the gradient where ribosomal RNA is eluted normally. The DNAase enzyme apparently leaves some DNA sufficiently polymerized to appear in its usual position. However, the amount of DNA present in the whole leaf must be greater than that in the chloroplast fractions. This level of DNAase should be adequate to depolymerize the DNA contained in the chloroplast fraction.

The presence of some residual RNA in the DNA/RNA peak would also contribute counts not arising from DNA. Messenger RNA associated with DNA would form an RNAase resistant system. The presence of this complex in the experimental material might negate previous results. To test for the

Fig. 16.-The distribution of OD260 and P^{32} incorporation into nucleic acid samples eluted from a methylated albumin column with a linear gradient of sodium chloride. One sample was treated with DNase for one hour; the other sample was not treated.

Twenty seedlings were labeled for 24 hours with 50 μ C P^{32} in 25 ml deionized water. Leaves were ground in omni-mixer with phenol. Phenol extracted nucleic acids were suspended in 10 ml saline-citrate.



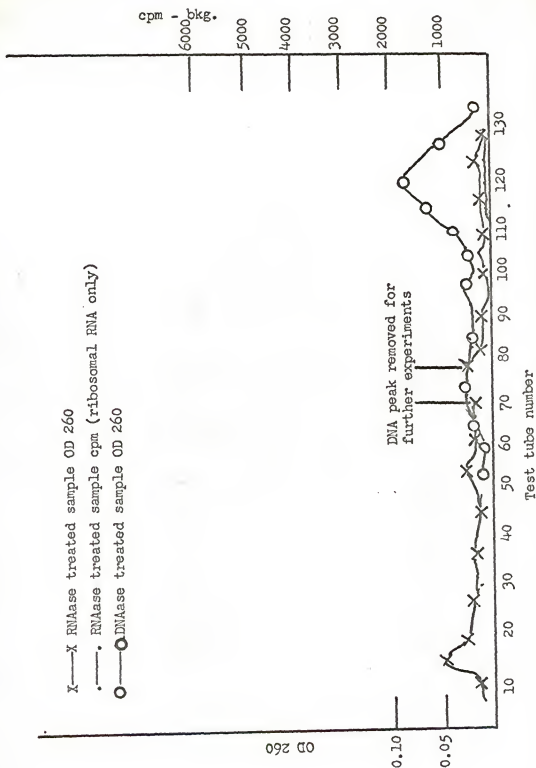


Fig. 17.—The distribution of OD 260 from RNAase treated nucleic acid extract in eluates from a methylated albumin column eluted with a linear sodium chloride gradient.

occurrence of this complex, the DNA/RNA peak from the RNAase treated sample was collected and subjected to alkaline hydrolysis. RNA is rendered acid soluble by this procedure. DNA acid insolubility is unaffected. Each collecting tube held 5 ml of eluate. Those tubes containing the major portion of the uv absorbance were selected and a 2 ml aliquot of sample was removed and dried on a planchet. The remaining portion of the eluate was treated with cold 1M perchloric acid and subjected to alkaline hydrolysis and all samples were counted at the same time. The results are shown in Table 8. If RNA were attached to DNA the supernatant fraction after basic hydrolysis should have a significant number of counts remaining in it. This was found to be true for several of the fractions. However, these results must be regarded as only preliminary since it is difficult to tell whether the radioactivity is due to RNA or suspended DNA. A comparison between the radioactivity recovered by alkaline extraction and that from the untreated portion of the sample reveals that a large amount of radioactivity was lost during the procedure.

A fresh sample of pea chloroplasts was prepared, and nucleic acids extracted from it. Detergent precipitate and detergent supernatant fractions were prepared. All

TABLE 8

THE INCORPORATION OF p^{32} INTO AN ALKALINE HYDROLYSATE OF RNAase TREATED NUCLEIC ACIDS ISOLATED FROM PEA LEAVES*

Fraction number	OD 260	Control cpm	Acid ppt cpm	Alk. hyd. cpm	Control cpm/5ml	Acid+alk. cpm/5ml
10	0.010	1				
11	0.020	1				
12	0.030	1				
13	0.020	2				
27	0.035	135	105	4	335	181
28	0.115	618	471	15	1545	810
29	0.175	671	494	48	1678	907
30	0.140	519	499	76	1295	961
31	0.097	347	348	21	868	615
32	0.074	209	137	18	525	258
33	0.041	114	104	1	295	156

*The samples to be hydrolyzed were extracted for 18 hours with 0.6M KOH at room temperature. The solution was acidified with 0.5M HClO₄ and the precipitate and supernatant fractions separated by centrifugation. The supernatant was dried on planchets. The precipitate was washed onto Millipore filter papers which were dried on planchets.

fractions were passed through methylated albumin columns without the addition of a marker DNA. A detectable amount of DNA was not washed off the column by 0.35M NaCl but was eluted with the second 50 ml of 1.0M NaCl. Table 9 shows the results when these washings were read on a DB spectrophotometer. A significant amount of uv absorbance was found in the eluates of the chloroplast detergent precipitate and the mitochondrial supernatant. It was then possible to study the DNA from the isolated chloroplast fractions without adding any other kind of DNA.

The major portion of the uv absorbance eluted from the methylated albumin column in the last experiment was collected and treated with DNAase. Before treatment, one of the samples was passed through the column to see if it would elute at the same position where it appeared on the first passage. As shown in Figure 18 the peak appeared on the same point as before, namely, when the second 50 ml of 1.0M NaCl buffer was added. After treatment with the enzyme, both samples were eluted with 0.35M NaCl. At the second fifty ml aliquot of 1.0M NaCl virtually no uv absorbance was eluted. If the material was DNA, treatment with this enzyme should give this result.

The procedure for preparation and separation of nucleic acids from chloroplast containing fractions was a bit long.

TABLE 9

THE DISTRIBUTION OF uv ABSORBING MATERIAL^a IN 1.0M NaCl
ELUATES FROM A METHYLATED ALBUMIN COLUMN TO WHICH
SAMPLES PREPARED FROM DETERGENT EXTRACTED CHLORO-
PLAST AND MITOCHONDRIAL FRACTIONS WERE ADDED^b

Test tube number	Chloroplast detergent precipitate	Chloroplast detergent supernatant fraction ^c	Mitochondria detergent precipitate	Mitochondria detergent supernatant fraction
	OD 260	OD 260	OD 260	OD 260
1	0.025	0.035	0.035	0.030
2	-	0.035	0.025	0.030
3	-	0.035	0.027	0.040
4	-	0.075	0.025	0.035
5	-	0.025	0.020	0.035
6	0.030	-	-	0.030
7	-	0.010	0.030	0.030
8	0.015	-	-	0.030
9	0.040	0.020	-	0.045
10	0.070	0.000	0.040	0.110
11	0.110 ^d	0.000	0.046	0.155
12	0.100 ^d	0.000	0.054	0.125
13	0.020	0.000	0.030	0.070
14	0.032	0.000	0.035	0.050
15	0.020	0.000	0.020	0.035

Table 9 (cont'd)

Test tube number	Chloroplast detergent precipitate	Chloroplast detergent supernatant fraction	Mitochondria detergent precipitate	Mitochondria detergent supernatant fraction
	OD 260	OD 260	OD 260	OD 260
16	-	0.000	0.020	0.035

^aThe uv absorbance is entirely due to DNA present in the material.

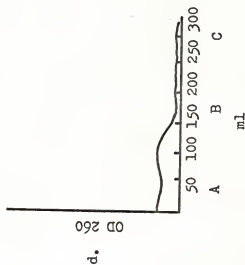
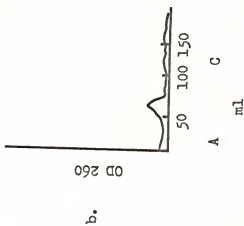
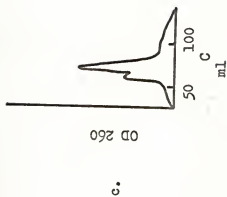
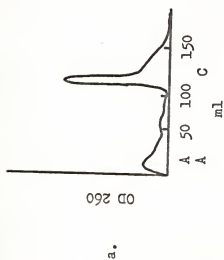
^bA group of 20 pea seedlings were labeled with 50 uc p^{32} in 25 ml deionized water. The labeling period was 24 hours of which 14 hours were in the light. Chloroplasts and mitochondrial fractions were extracted with detergent. The detergent supernatant fraction was concentrated by freeze drying. All samples were extracted with phenol and treated with RNAase for one hour.

^cTwo ml of tubes 8, 9, 10, 11, 12, and 13 of chloroplast detergent supernatant were collected and counted. Only tube 11 showed a significant number of counts above background. It was 7 cpm above background. BKG equalled 17 counts per minute.

^dMaximum absorption point at 260.

Fig. 18.-The effect of DNAase on the elution pattern of a RNAase resistant nucleic acid from a methylated albumin column.

- a. A uv absorbance tracing of an eluate from a methylated albumin column of detergent precipitated chloroplast DNA previously treated with RNAase for one hour.
- b. A uv absorbance tracing from a methylated albumin column of the 1.0M NaCl eluate from run shown in (a). The sample was extracted with DNAase for one hour.
- c. A uv absorbance tracing from a methylated albumin column of detergent supernatant mitochondrial preparation previously treated with RNAase for one hour.
- d. A uv absorbance tracing from a methylated albumin column of the 1.0M eluate from the run shown in (c). The sample was treated with DNAase for one hour.



Legend: A = 0.35M NaCl

B = 0.50M NaCl

C = 1.0M NaCl

An attempt was made to shorten the process by leaving out the elution of the sample from a methylated albumin column. To remove RNA from DNA an alkaline hydrolysis procedure was used. Since the samples were counted rather than studied by means of their uv absorbances, the presence of a small amount of detergent which absorbs heavily at 260 should not interfere with the determination of the relative amounts of p^{32} incorporation into DNA. The chloroplast and mitochondrial fractions were prepared and extracted with detergent. The acid precipitates of each fraction were hydrolyzed in alkali and after acidification the supernatant fraction was separated from the precipitate. Both were collected and counted in the usual manner. The results of this experiment are presented in Table 10. More counts were retained in these fractions than were retained by previous techniques. Several explanations are possible for this. The precipitates counted are not only DNA but may contain protein and cell wall material. In addition, in spite of careful washing with lipid solvents some phospholipid may be retained. The presence of phosphoprotein also cannot be ruled out. The light and dark treatment both developed some chlorophyll. The covering over the seedlings supposedly grown in the dark was not sufficient to exclude all light. The great differences in incorporation between the light and supposedly dark grown samples remained

TABLE 10

THE INCORPORATION OF P^{32} * INTO ALKALI INSOLUBLE AND SOLUBLE PORTIONS OF DETERGENT EXTRACTED MITOCHONDRIAL AND CHLOROPLAST FRACTIONS** ISOLATED FROM ETIOLATED PEA LEAVES*** EXPOSED TO LIGHT AND DARK ****

	Alkali insoluble cpm above bkg	Alkali soluble cpm above bkg
Detergent precipitate		
<u>light treated</u>		
chloroplast	76	142
mitochondria	441	720
<u>dark treated</u>		
chloroplast	133	190
mitochondria	720	879
Detergent supernatant fraction		
<u>light treated</u>		
chloroplast	25	57
mitochondria	206	292
<u>dark treated</u>		
chloroplast	41	361
mitochondria	141	87

* 50 uc P^{32} in 25 ml deionized water for 24 hours.

** Chloroplast and mitochondrial fractions suspended in 40 ml 0.01M Tris buffer pH 7.7 containing 0.01M Mg^{++} ; 0.5 ml of 5 per cent deoxycholate added to the suspension. Centrifuged at 30,000 X g for one hour.

*** Two batches of etiolated seedlings, 20 each, were excised from roots. One batch was exposed to 14 hours of light, the other was grown in the dark under a beaker covered with Al foil placed in the same growth chamber as the light grown seedlings.

Table 10 (cont'd)

****All fractions were treated with an equal volume of cold 1M HClO_4 and the acid precipitates extracted with absolute ethanol. Alkaline hydrolysis was for 18 hours at room temperature using 0.6M KOH. The hydrolysate was acidified and the precipitate separated from the supernatant by means of a 30,000 X g centrifugation. The supernatant fraction was dried in stainless steel planchets. The precipitate was washed into planchets with acetone and dried.

unexplained since both apparently got enough light to begin the manufacture of chlorophyll. These results cannot be simply explained by differences in light intensity even though the shaded samples must have had less intense light than the light-grown samples. The determination of the amount of chlorophyll synthesized in the samples would have been an aid in resolving this question. Protein also should have been determined because the size of the samples might have been quite different. The larger incorporation of isotope into the mitochondrial fraction is explainable by assuming that the chloroplasts have not reached their most mature stage of development. The developing chloroplasts are usually less massive than the mature chloroplasts, hence it takes a higher centrifugal force to sediment the younger plastids.

It was interesting to compare the P^{32} incorporation of plants growing under darkened conditions and those starting to green. To determine if the greening procedure involved a synthesis of chloroplast DNA, two groups of seedlings were grown, one exposed to light for six hours and the other in total darkness. The 3,000 X g centrifugation was omitted since few plastids from etiolated plants would sediment at this speed. The 30,000 X g precipitate was extracted with detergent after the usual sucrose gradient

centrifugation. Methylated albumin chromatography was performed after phenol extraction and RNAase digestion. Only the 1.0M eluate was collected for counting. Both light and dark treated samples were counted at the same time. The samples were approximately the same size. The data in Table 11 indicate that there was little difference in the incorporation of P^{32} into the chloroplast precipitate. The distribution of radioactivity is probably not significant since this depends on many uncontrolled factors.

The detergent supernatant fractions were not concentrated on a freeze dryer or extracted with RNAase. Instead, the entire 100 ml of the sample was placed on a methylated albumin column which was washed with the buffer suspending the nucleic acids. Although the supernatant fraction was buffered at pH 7.7 and the column is ordinarily run at pH 6.7 the change in pH did not seem to affect the retentative ability of the column since the second fifty ml of 1.0M NaCl also eluted the major portion of the uv absorbing material as usual. As shown in Figure 19, similar peaks occurred in extracts from both light and dark grown plants. The two supernatants were mixed together and treated with DNAase for one and one-half hour. The results, shown in part (c) of Figure 19, demonstrated that both DNA and RNA were present in the samples. The DNA was elutable

by a low ionic strength buffer (0.35M NaCl leaving a uv absorbance peak still attached to the column. This peak must be either a RNA or an unhydrolyzed DNA. Previous experiments with DNAase indicated that this enzyme seldom left appreciable amounts of DNA undegraded. The presence of RNA in extracts from chloroplasts isolated from dark-grown plants was reported earlier in Table 1 and Figure 3. The methylated albumin column may also be used to concentrate nucleic acid suspensions when the material is suspended in large volumes of liquid.

CHARACTERIZATION OF THE DNA ISOLATED

CsCl gradient centrifugation. The central problem remaining was the identity of the DNA fractions prepared by the detergent treatments. Were the DNAs isolated from the detergent precipitate and from the detergent supernatant the same or different molecules? To answer this question two approaches were undertaken.

Different species of DNAs may be separated by density gradient centrifugation. CsCl solutions may be prepared which approximate the density of most DNA molecules. Such solutions when centrifuged rapidly for a day at constant temperature have established in them a concentration of gradient. Foreign molecules will distribute themselves in the gradient according to their buoyant densities. A relationship exists between the buoyant density of the

TABLE 11

INCORPORATION OF P^{32} * INTO THE DETERGENT PRECIPITATES**
ISOLATED FROM THE 30,000 X g PRECIPITATE PREPARED FROM
LEAVES OF ETIOLATED PEA PLANTS*** EXPOSED TO SIX HOURS OF
LIGHT OR TO DARKNESS

Fraction from methylated albumin column	Dark grown plants cpm above bkg	Light grown plants cpm above bkg
9	2	4
10	8	3
11	11(s)	2
12	7	11(s)
13	1	4
(s) = significant		

* Labeled with 30 μ C P^{32} in 25 ml deionized water for 26 hours.

** The 30,000 X g precipitate was suspended in 100 ml of Tris buffer containing 0.01M Mg^{++} . Ten ml 5 per cent sodium deoxycholate was added and the suspension was centrifuged for one-half hour at 30,000 X g. Phenol extraction, alcohol precipitation and methylated albumin chromatography were performed. The 1.0M NaCl eluates were collected and counted.

*** The leaves were homogenized in a Brender homogenizer.

molecule and its base composition. Using this technique it is possible to separate two species of DNA and calculate the base composition of each. (Vinograd and Hearst 1962).

Another method of characterizing DNA requires molecules of a single species to be present. Contaminants effect the results in unforeseen ways. DNA is a double stranded molecule whose two complimentary parts are held together by hydrogen bonds existing between purines and pyrimidines in different strands. One pair, the guanine - cytosine (G-C) pair has one more hydrogen bond than the adenine - thymine pair. Heating solutions of the molecule cause the unfolding of the strands - the so called melting phenomenon. Molecules containing higher guanine - cytosine contents than other molecules will have higher melting point ranges than molecules of lower G-C content. The mid-point of the melting point range is taken as a convenient measure of this range. Marmur and Doty (1962) used a large number of bacterial DNAs to derive a relationship between melting temperature and base composition.

CsCl gradient centrifugation

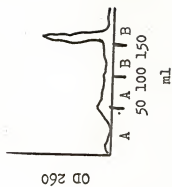
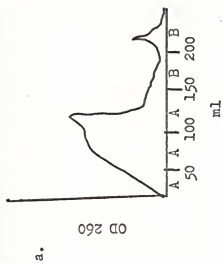
A sample of E. coli DNA was added to the top of a preformed CsCl gradient. The sample was centrifuged for 12 hours at 120,000 X g at 19°. At the end of this time

Fig. 19.-A uv absorbance tracing from a methylated albumin column of a detergent supernatant prepared from the 30,000 X g precipitate of an homogenate prepared from light grown and dark grown pea plants.

- a. A uv absorbance tracing from a methylated albumin column of a supernatant fraction prepared from dark grown peas exposed to six hours of light in a growth chamber. The light was from cool white fluorescent bulbs supplemented with incandescent light. The sample was not treated with phenol or RNAase.
- b. A uv absorbance tracing from a methylated albumin column of detergent supernatant prepared from dark grown peas unexposed to light. The sample was not treated with phenol or RNAase. The peaks obtained were the same as in (a). The large peak eluted by 0.35M NaCl is deoxycholate since it is insoluble in acid but soluble in alcohol, a solvent which does not dissolve nucleic acids.
- c. A uv absorbance tracing from a methylated albumin column of the 1.0M eluate from (a) and (b) above, treated for one and one-half hours with DNAase.

A = 0.35M NaCl

B = 1.0M NaCl



the sample was divided into equal parts and read on a Beckman DB spectrophotometer fitted with a microcuvette. The results of this experiment are shown in Table 12. E. coli DNA bands at one particular spot in the gradient. The density of the solution at this point is close to the buoyant density of E. coli DNA, 1.710, reported by Schildkraut, Marmur, and Doty (1962). E. coli DNA was used in these preliminary experiments because its buoyant density is known. The position of E. coli DNA in the gradient would form a spot of known density from which assuming linearity of gradient it would be possible to calculate densities of other molecules. Other experiments were done using pea and oat DNAs but the results were inconclusive.

The long periods of centrifugation at high speed and the difficulties in handling the easily disturbed density gradients made this technique less advantageous than the apparently simpler melting point procedure. Since a technique had already been worked out for the purification of the chloroplasts, fairly pure chloroplast DNA should be obtained. The melting point procedure was adopted as the chief means of characterizing the DNA.

TABLE 12

THE DISTRIBUTION OF OPTICAL DENSITY AT 260 m μ DUE TO E. coli
DNA THROUGH A CsCl GRADIENT

Sample number	OD 260	Pt. max. absorbance	Buoyant density*
27	0.250		
26	0.195		
25	0.315	270	
24	0.205	plateau 270	
23	0.195	plateau 270	
22	0.200	plateau 270	
21	0.230	plateau 270	
20	0.270	plateau 270	
19	0.290	plateau 270	
18	0.269	plateau 270	
17	0.241	plateau 270	
16	0.195	plateau 240-260	
15	0.290	plateau 240-260	
14	0.285	plateau 240-260	
13	0.270	plateau 240-260	
12	lost	-	
11	0.358	-	1.6318
10	lost	-	-
9	1.00	260	1.6970
8	0.340	no peak 260	-
7	0.270	no peak 260	1.6318
6	0.319	no peak 260	1.6839
5	0.229	no peak 260	1.6025
4	0.250	no peak 260	1.5834
3	0.248	no peak 260	1.5840

Table 12 (cont'd)

Sample number	OD 260	Pt. max. absorbance	Buoyant density*
2	0.248	no absorbance at 260	1.5775
1	0.240	no absorbance at 260	1.5645

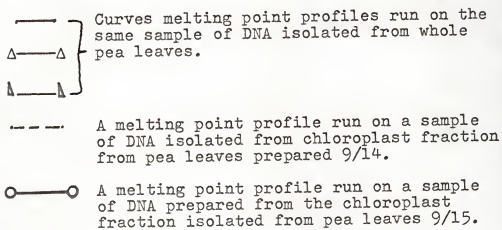
*The buoyant density of certain fractions was calculated by measuring the index of refraction of the fraction in a refractometer.

**A CsCl gradient was prepared using a gradient maker. Three ml of a CsCl solution of buoyant density 1.800 were added to the cylinder containing the effluent valve. Three ml of a CsCl solution of buoyant density 1.600 were added to the other cylinder. After the gradient was formed, two ml of CsCl solution of buoyant density 1.800 were added to the very bottom of the gradient to prevent the sample from binding in the hemispherical portion of the tube. Two ml of CsCl solution of density 1.600 were added to top of gradient. The solution contained *E. coli* DNA. The gradient was centrifuged at 120,000 X g for 12 hours at 19°C in head 50 of the Spinco Model L preparative ultracentrifuge. At the end of this time, the tube containing the gradient had a hole punched in its bottom with a tube punching apparatus and five drops of the gradients were collected into each collecting tube. The OD 260 of each tube was read on a Beckman DB spectrophotometer against a water blank. A microcuvette with an effective volume of 0.3 ml and a light path of 0.1 cm was used to read the samples.

Melting Point Determinations

Deoxyribosenucleic acids isolated from whole pea leaves and from the mitochondrial and chloroplast fractions were heated in a cuvette in a Beckman DU spectrophotometer. The temperature of the chamber was raised to about 75°C. Each temperature regime was maintained for about ten minutes. At the end of this time the control on the thermoregulator of the circulating water bath was rotated to give a rise within the cuvette of approximately 2°F. OD readings were expressed as relative increase in optical density (RIOD) obtained by dividing the optical density reading at a temperature by one obtained at a standard temperature usually room temperature. The data obtained were plotted on a curve as in Figures 20 and 21. In Figure 21 the curves are not plotted against the same RIOD values in each curve. The RIOD values have been so chosen to minimize the distances of the lowest RIOD value from the x axis. This procedure was adopted in calculating the midpoint of the melting point profile curves. The increase in optical density observed occurs over approximately the same range of temperature. This suggests that the same molecule is involved in the denaturation. Variation in relative increase in optical density was due to variations in the reading at the standard temperature which sometimes was higher in value than the readings at

Fig. 20.-Melting point profiles of pea DNAs.



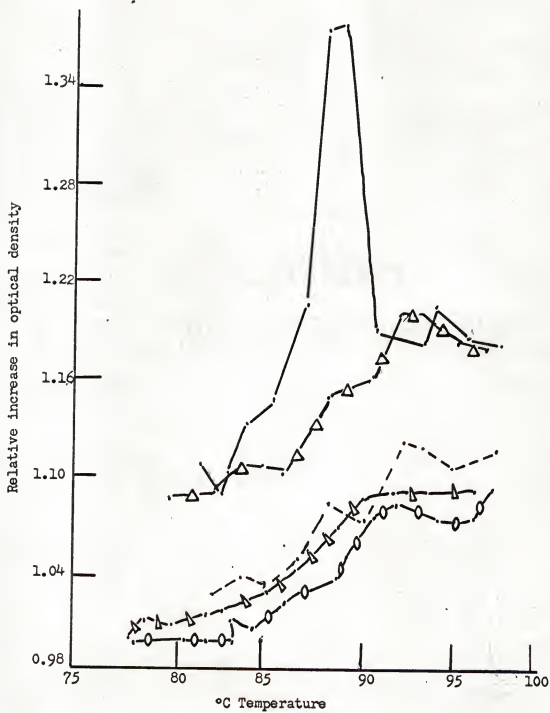
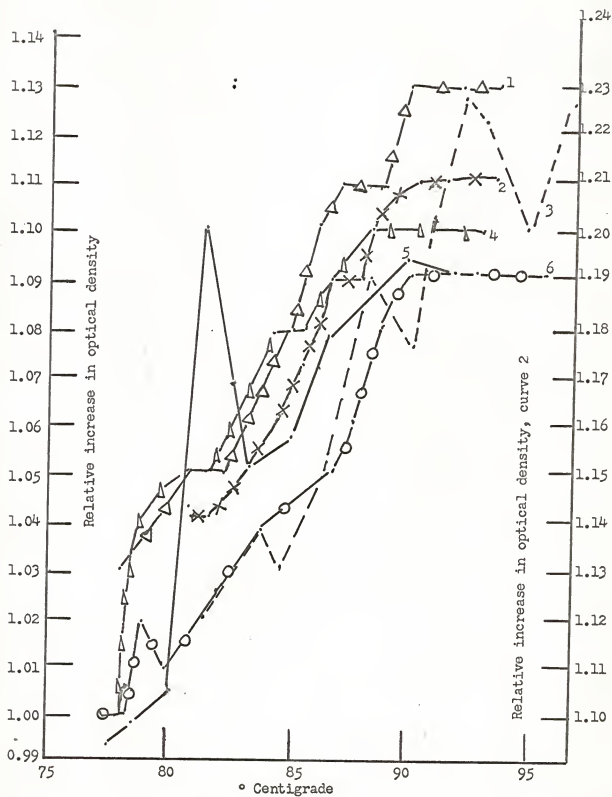


Fig. 21.-Melting point profiles of DNAs prepared from mitochondrial and chloroplast fractions from pea leaves.

Curve 1 - Chloroplast fraction	8/4	Δ — Δ
Curve 2 - Mitochondrial fraction	8/5	
first experiment		\times — \times
Curve 3 - Chloroplast fraction	9/14	— — —
Curve 4 - Mitochondrial fraction	8/5	
second experiment		Δ — Δ
Curve 5 - Mitochondrial fraction	8/11	— — —
Curve 6 - Chloroplast fraction	9/15	\circ — \circ



higher temperatures. This led to fractional values for RIOD. These could not be plotted on the same paper as the majority of other values without assigning these low values to the same axis and plotting the points using these values. This procedure led to the discovery that in spite of the variations in RIOD values the curves often resembled each other in form.

In calculating the midpoint of the denaturation curve the following assumptions were made:

- a. The same DNA is present in both mitochondrial and chloroplast fractions.
- b. The melting point curves for this DNA are sigmoid in nature and resemble those given by Marmur and Doty (1962) for E. coli DNA.

- c. A single curve could be fitted to the points making up the five curves of Figure 21. The middle portion of this curve would be approximated by a straight line.

The points between the temperatures 81°C and 88°C were chosen because they are most likely to lie on the linear portion of the curve as inferred from the experimental results. A straight line was fitted to these points by the method of least squares. The midpoint of the melting point curve could be estimated by taking the point of the line segment between the two linear horizontal portions of the melting point profile. The data were plotted so that the

lowest value would be the x axis. The upper value was calculated by calculating the average value of y at the three highest temperatures, values which were clearly in the horizontal portion of the curve. The melting point of the DNAs isolated from the mitochondrial and chloroplast portion was 84.8°C and corresponds to a GC composition of 37.76 mole per cent.

In Figure 20, curves 1, 2, and 5 are curves drawn from the same sample of DNA isolated from whole pea leaves. Curves 3 and 4 are melting point curves of chloroplast fraction DNA isolated from pea. The melting point range of the curves were approximately the same suggesting that they are the same molecule. Hence the DNA base composition cited above must be indicative of pea nuclear DNA.

DISCUSSION

Deoxyribosenucleic acid was demonstrated to be present in chloroplast and mitochondrial fractions from pea leaves. The demonstration depended upon the removal of all uv absorbing substances other than DNA. Deoxyribosenucleic acid was detected by its resistance to RNAase treatment, its position of elution on a methylated albumin column, its uv absorption spectrum and most of all by its depolymerization upon treatment with DNAase.

Deoxyribosenucleic acid from homogenates of plant cells may arise from several sources other than chloroplasts. Some of these are the nucleus, the mitochondria, and chance microbiological contaminations. It is necessary to show that the DNA isolated from an homogenate differs in base composition from nuclear DNA of the same plant. Microbiological contaminations would be harder to rule out because of the difficulties of establishing an aseptic culture of large plants. Most microbiological populations on the surfaces of healthy plant leaves are small and this possible source of contamination is, therefore, unlikely to be detected. Mitochondria are difficult to separate from plastids broken during the isolation procedure. For this reason it is difficult to interpret results

showing a different base composition than nuclear in mitochondria containing fractions. Edelman, Schiff, and Epstein (1965) reported the presence of DNA in the mitochondria of Euglena. They found that it was necessary to utilize radioisotopic technique to locate the mitochondrial DNA since it was present in such small quantities. The chloroplast DNA was detected by optical means. The detectability of the mitochondrial DNA by optical means depends on using enough material. Since optical methods for the most part were used in the detection of plastid DNA, the possibility of mitochondrial DNA contamination is a minor one. Phosphorus count incorporation would undoubtedly not distinguish between the two forms.

In order to prepare pure chloroplast DNA some method would have to be used to separate the DNA species from each other. Methylated albumin chromatography, so useful in the separation of RNA species and RNA and DNA is not useful for this purpose as shown by the close coincidence of P^{32} counts incorporated into pea DNA and the OD 260 peaks of corn DNA. CsCl gradient centrifugation makes it possible to separate the mitochondrial, chloroplast and nuclear DNA.

Another possible way to demonstrate the presence of different DNAs in the same cell is the calculation of the melting point of isolated DNA from melting point profiles run under standard conditions. The DNA melting point calcu-

lated from extracts of whole leaves would be characteristic of nuclear DNA since this is the species of DNA present in the highest quantity in cells. Isolation and purification of chloroplast fractions would reduce the amount of nuclear DNA in the fraction and change the calculated melting point to one more characteristic of chloroplast DNA.

A method discovered by Brawerman and Eisenstadt (1964) can be used for separating the nuclear DNA from chloroplast DNA without using CsCl gradient centrifugation. The chloroplast fraction was extracted with sodium deoxycholate. The bounding membranes of the chloroplast disintegrate under this treatment as can be seen when detergent is added to plastid suspensions under the microscope. The internal membranes of the photosynthetic lamella system are unaffected by this treatment since most of the chlorophyll which is membrane bound is sedimentable at 30,000 X g. A DNA was sedimented along with these plastid fragments. It had a base composition differing from nuclear DNA and appeared at the same buoyant density level in a CsCl gradient to which chloroplast DNA was sedimented in previous experiments. A DNA of base composition resembling the nuclear one was recovered from the supernatant by a 100,000 X g centrifugation. Apparently the internal membranes of the plastid prevent the release of the chloroplast DNA to the supernatant. Somewhat similar results were reported by Iwanmura (1960). Chlorella

cells were exploded by placing them in a French press under high pressure and suddenly releasing the pressure. The external membranes of the plastid were destroyed by this procedure but a DNA was recoverable from the plastid fragments sedimenting at 20,000 X g. When the supernatant was sedimented at 105,000 X g a DNA of different base composition was found. Iwanmura concluded that the DNA associated with the plastid fragments was chloroplast DNA and that found in the supernatant fraction was nuclear DNA. Ris and Plaut (1962) observed that the DNAase sensitive areas in the sections chloroplasts of Chlymydomonas observed under the electron microscope were completely surrounded by the internal lamella of the plastid. Recently Bezinger, Molchanov, and Sissakin (1966) observed that a nucleic acid was associated with the isolated lamella system of kidney bean chloroplasts. These results indicate that the chloroplast DNA must be somehow associated with the lamella system of the plastids. It is then possible to prepare bounding membraneless plastids which still retain their DNA. The evidence is also good that the nuclear particles are much less stable to these treatments and rapidly lose their material to the external medium. Stable nuclei are exceedingly difficult to prepare.

Both the detergent extracted plastid and mitichondrial fractions show DNA in the supernatant portion and in the

precipitate. Although a DNA does not sediment when the detergent supernatant fraction of peas is sedimented at 100,000X g as is the case in Euglena, evidence would suggest that a similar situation occurs in the detergent extraction of pea chloroplasts. Further characterization of the DNA associated with the two fractions would have to be made before a final decision could be made.

A slight amount of P^{32} was incorporated into the plastids during the 24-hour labeling period. This indicates that chloroplast DNA synthesis is proceeding. It is unknown whether the plastid division continues with cessation of cell division in a mature plant cell. In the rapidly elongating internodal cell of Nitella (Green, 1964), chloroplast division still proceeds after cell division has stopped. No evidence was found on this point.

SUMMARY

Deoxyribosenucleic acid was isolated from chloroplast containing cell-free fractions prepared from pea leaves. The DNA was freed from protein and lipid by a phenol extraction procedure. Ribosenucleic acid was removed by hydrolysis with RNAase and passage through a methylated albumin column. The hydrolyzed RNA was washed off the column with low ionic strength buffers. The unhydrolyzed DNA remained on the column and was eluted by buffers of higher ionic strength. The DNA was depolymerized by treatment with DNAase. The major portion of the DNA liberated and isolated by the fractionation procedures occurred in the chloroplast containing fractions. On treating the isolated fractions with 5 per cent sodium deoxycholate, a portion of the DNA was not sedimented with the chloroplast fragments. It was found in the supernatant fraction and was not sedimented by a 2-hour centrifugation at 100,000 X g. Another portion of the DNA was sedimentable with the plastids after detergent treatment. Attempts to characterize these different fractions were not successful.

A small amount of P^{32} was incorporated into the DNA of the chloroplast containing fractions. This was only a small portion of the total P^{32} incorporated into the DNA of the leaves.

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BIOGRAPHICAL SKETCH

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 13, 1966


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